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The Horseshoe Crab *Tachypleus tridentatus* has Two Kinds of Hemocytes: Granulocytes and Plasmatocytes

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Abstract. For the first time, the fine structure of the hemocytes from the horseshoe crab *Tachypleus tridentatus* is investigated by transmission electron microscopy and light microscopy serial sectioning. Two morphologically distinct, ellipsoidal, and mononucleate hemocytes—granulocytes (amebocytes) and plasmatocytes—are revealed. Granulocytes constitute about 97% of the hemocytes. They have a marginal band of microtubules, a heterochromatic nucleus, distended but poorly developed RER, few free ribosomes, few mitochondria, and many large secretory granules. The majority of these granules have a uniform content and are mature. Structured granules located in the proximity of Golgi complexes may be immature transitional stages leading to the mature uniform granules. Upon stimulation with endotoxin from gram negative bacteria, the mature granules become transitory structured before exocytosis. In contrast, the immature granules are not exocytosed. Plasmatocytes constitute about 3% of the hemocytes. They differ from granulocytes by having an euchromatic nucleus, a well-developed RER of flattened or tubular cisternae, many free ribosomes, many mitochondria, but only few, if any, large secretory granules. Apparently, plasmatocytes are not affected by endotoxin. The relationship and possible functions of granulocytes and plasmatocytes are discussed and compared with those of the horseshoe crab, *Limulus polyphemus*.

Introduction

Horseshoe crabs are "living fossils," which have undergone little morphological evolution during the last 360 million years; they can be traced back more than 500 million years (Sekiguchi and Sugita, 1980; Shishikura *et al.*, 1982; Mikkelsen, 1988). If this stability is reflected in their physiology, studies of their immune defense system may shed light on when and how the different parts of it evolved in horseshoe crabs and possibly also in higher and more recent phyla.

Inoculation of gram negative bacteria or their endotoxins into the hemolymph of horseshoe crabs cause fatal intravascular coagulation (Bang, 1956). This involves exocytosis of the large secretory granules from the hemocytes. These granules contain coagulogen and all other proteins necessary for the coagulation (Levin and Bang, 1964; Ornberg and Reese, 1981; Iwanaga *et al.*, 1986; Suhr-Jessen *et al.*, 1989). Hemocyte (amebocyte) lysates can be made from all four extant species of horseshoe crabs, and are now extensively used to detect minute quantities of endotoxin (Shishikura *et al.*, 1983; Watson *et al.*, 1987).

The horseshoe crab best characterized is *Limulus polyphemus*. Until recently, only one hemocyte, the granulocyte, had been identified in this species (Dumont *et al.*, 1966; Levin and Bang, 1968; Copeland and Levin, 1985; Tablin and Levin, 1988). However, a second hemocyte, the plasmatocyte, has been identified independently by light microscopical observations of live cells, by light microscopical serial sectioning of fixed cells, and by transmission electron microscopy alone and combined with immuno-gold labeling (Suhr-Jessen *et al.*, 1989). In addition, cyanocytes and cyanoblasts have been reported to be present in the sinusoids around the compound eyes (Fahrenbach, 1970). Early light microscopical studies suggested that *Tachypleus tridentatus* had two kinds of granulocytes (Shishikura *et al.*, 1977; Shishikura and Sekiguchi, 1979).

The aim of the present study is to characterize the fine structure of *T. tridentatus* hemocytes—the cellular part of the immune defense system—in the pres-

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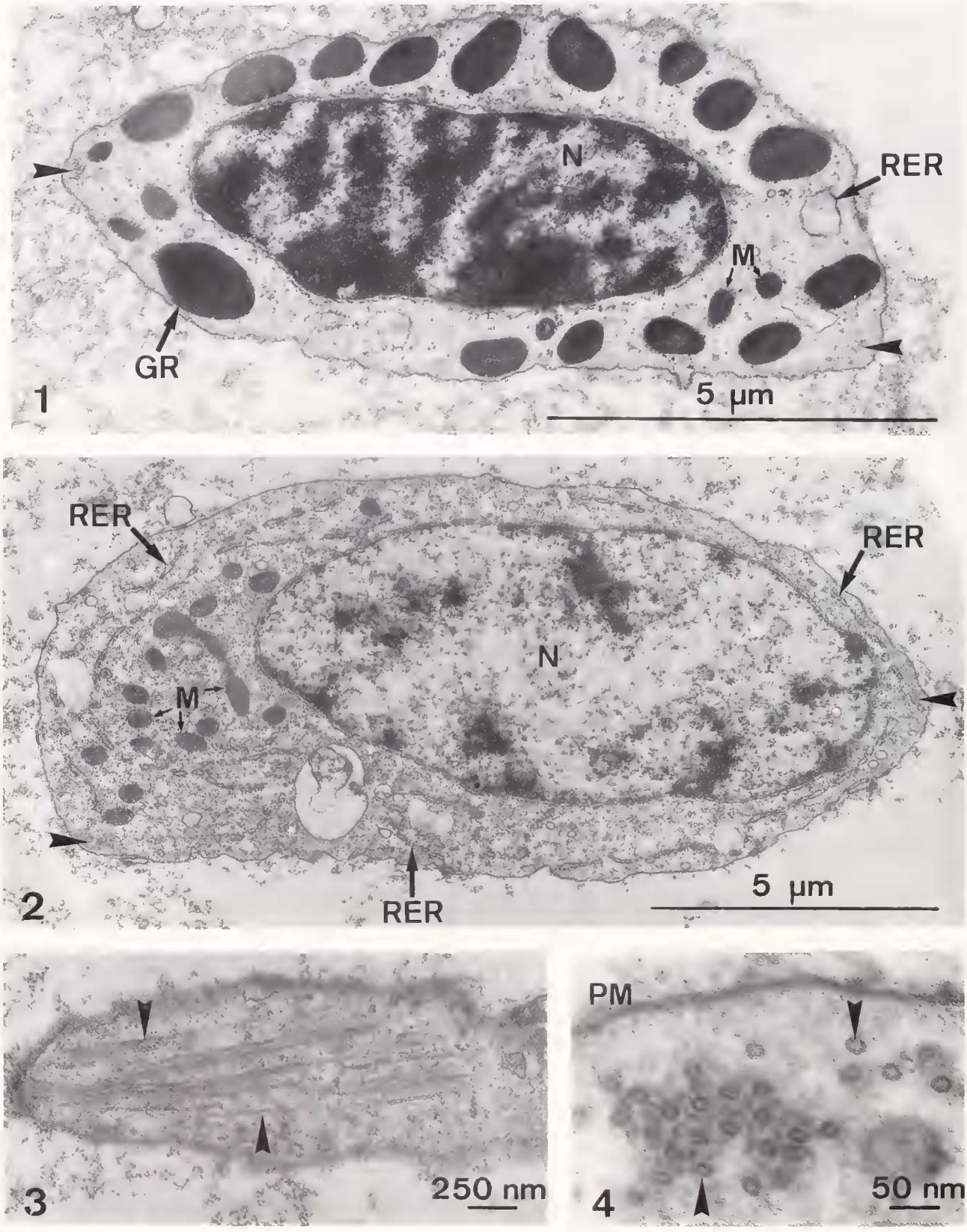
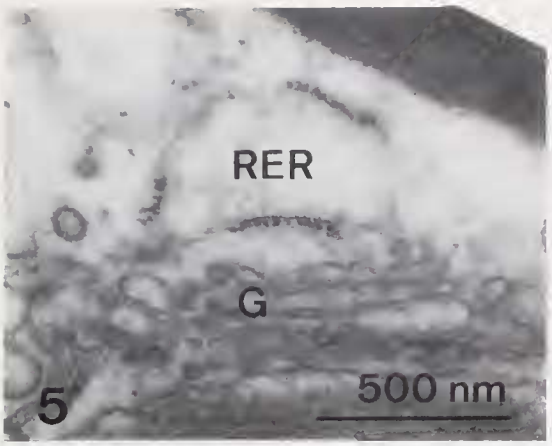
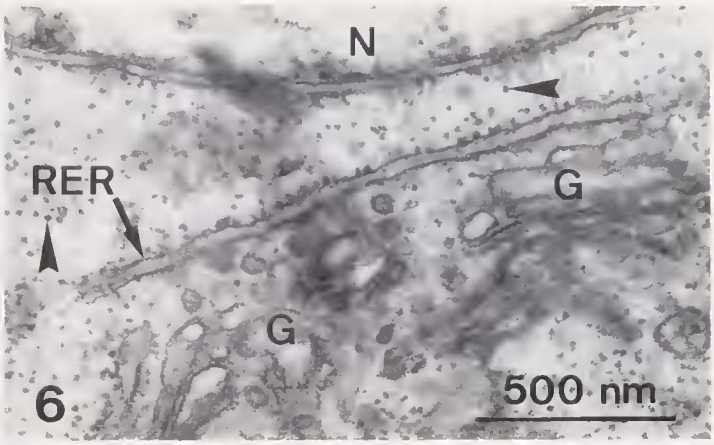


Figure 1. *Tachypleus tridentatus* granulocyte with its heterochromatic nucleus (N), and many large secretory granules (GR). Mitochondria (M). Rough endoplasmic reticulum (RER). Marginal band (arrowheads).





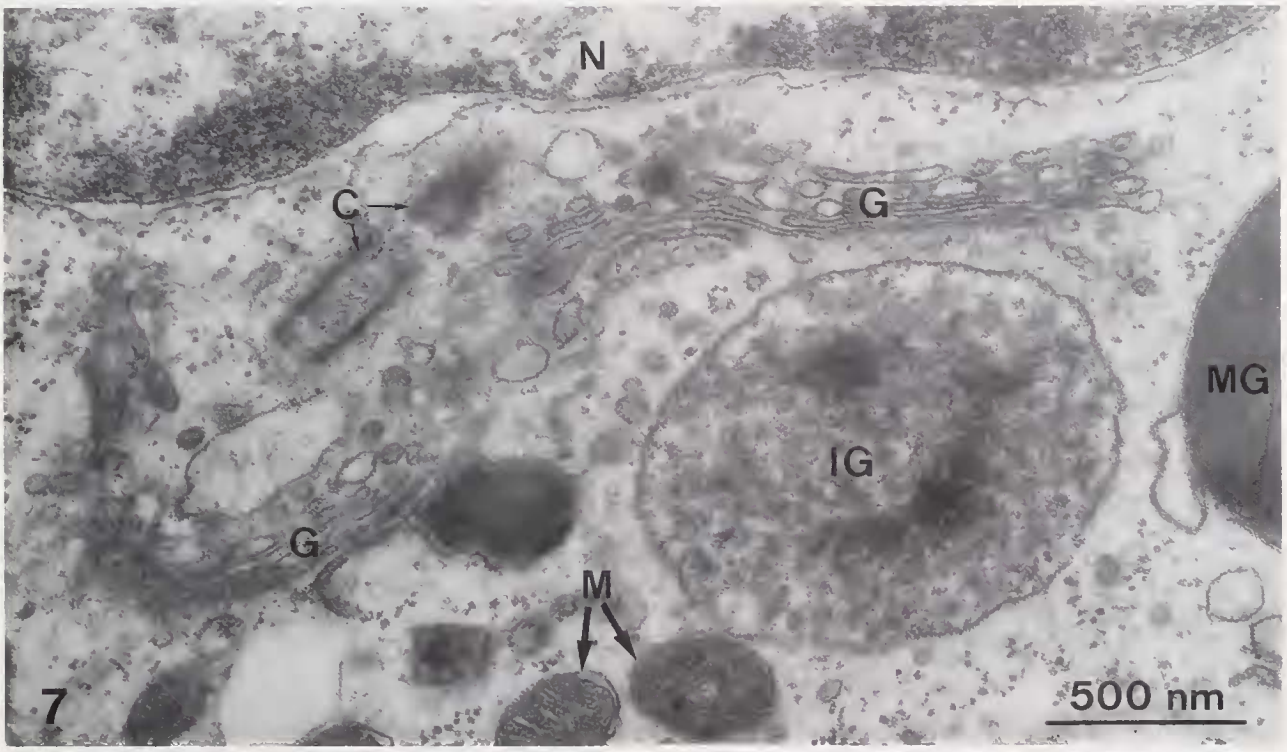


Figure 5. The distended rough endoplasmic reticulum (RER) from a *Tachypleus tridentatus* granulocyte. Golgi complex (G).

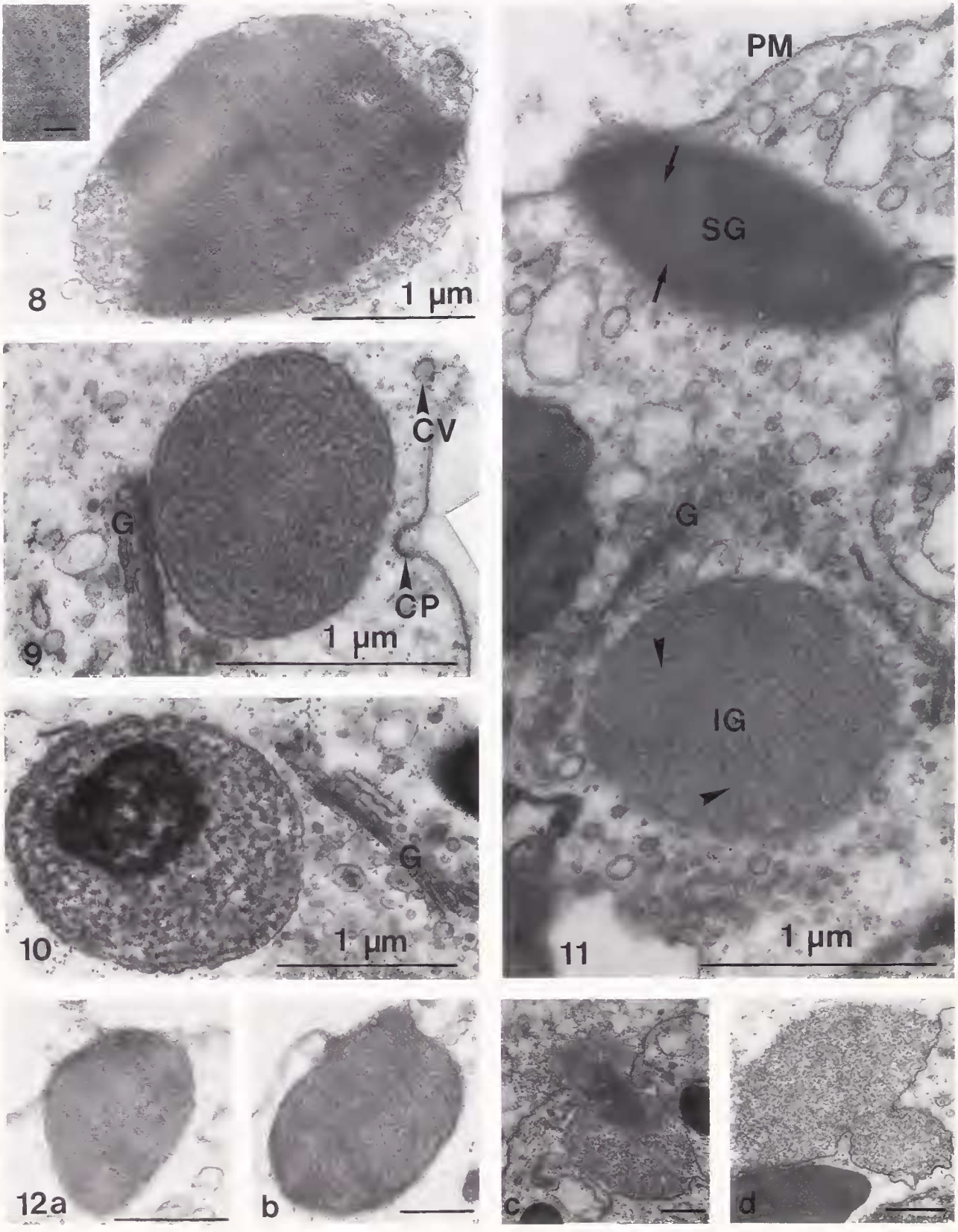
Figure 6. The flattened or tubular RER from a *T. tridentatus* plasmatocyte. Many free ribosomes are present (arrowheads). Golgi complex (G); nucleus (N).

Figure 7. *T. tridentatus* granulocyte with a structured (immature) large granule (IG) located in close proximity to a Golgi complex (G). Centrioles (C); mitochondria (M); uniform (mature) granule (MG); nucleus (N).

ence and absence of endotoxin. We show that the general circulation of *T. tridentatus* contains plasmatocytes and a single class of granulocytes. Furthermore, a temporal relationship is described for the formation to final secretion of the large secretory granules in the granulocytes.

Figure 2. *T. tridentatus* plasmatocyte with its euchromatic nucleus (N), well-developed RER, and many mitochondria (M). Marginal band (arrowheads).

Figures 3, 4. Longitudinal and transverse sections of marginal bands of microtubules (arrowheads) in *T. tridentatus* hemocytes. Plasma membrane (PM).



Figures 8–10. Differently structured immature large granules from *Tachypleus tridentatus* granulocytes. Insert: close-up of the about 17-nm tubular structures in transverse and longitudinal section (bar equals 100 nm). Apparently, a coated pit (CP) and a coated vesicle (CV) are present. Golgi complexes (G).

Materials and Methods

Six adult *T. tridentatus* females (males were not available) (prosomal width: 30–33 cm) were collected in the Tonkin Gulf, China, and kept in seawater (3.0% NaCl) at 15°C at The Danish Aquaculture Institute, Hørsholm, for up to nine months. Throughout this period, hemolymph samplings from all animals gave similar results. Hemolymph was drawn by cardiac puncture at the ethanol-cleaned prosoma-opistosoma junction. Access to the heart was made by a 19-gauge needle alone or combined with a 5-ml syringe containing fixative or, as part of a total bleed of the animal, through a large cut by a sterile (LPS-free) scalpel. The three methods gave similar results. Hemolymph was floating directly into 5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4, to give a final glutaraldehyde percentage of no less than 4. Samples were also incubated for 5 to 300 s with 10^{-4}–10^{-13} g *E. coli* endotoxin (Sigma no. L 3755)/ml hemolymph prior to fixation. The fixed samples were processed as described (Willumsen *et al.*, 1987). Transmission electron microscopy sections (about 50 nm) were mounted on pioloform F-50 coated Cu- or Ni-grids, contrasted with lead citrate, examined in a Jeol JEM-100CX electron microscope at 80 kV, and photographed using Agfa-Gevaert 23D56 film. Light microscopy serial sections (about 1.0 μm) were stained with toluidin blue, examined in a Zeiss microscope (numerical aparture: 1.30) at 400× using immersion oil, and photographed using Kodak panatomic X film. To eliminate inaccuracies due to minor differences in the thickness of the sections, a plasmatocyte was always compared with a nearby granulocyte starting and ending at almost the same section numbers. In the two cells, the number of cuts through mitochondria rather than the actual number of mitochondria was determined. Assuming that the mitochondria are randomly oriented and approximately of the same size in the two cells, any consistent deviation from 1 in the PL/GR ratio reflects differences in numbers of mitochondria.

Results

General morphology of the hemocytes

T. tridentatus hemocytes are spheroid, and about 15–20 μm at their longest axis (Figs. 1, 2, 15). A marginal band of microtubules run parallel to the longitudinal axis of the cells at least one microtubule diameter beneath the plasma membrane (Figs. 1–4). The almost parallel arrangement of the microtubules, combined with the electron-dense material seen between them, suggest that they are connected (Figs. 3, 4). Each hemocyte has a single, non-lobated nucleus containing one or a few nucleoli. The cells also contain rough endoplasmic reticulum (RER), free ribosomes, mitochondria with lamellar cristae, and Golgi complexes with 3–6 layers of cisternae—the cis-ones being more distended than the trans-ones (Figs. 1–2, 5–7). The paired centrioles form an obtuse angle to each other (Fig. 7). No sign of mitosis was seen in any of the examined hemocytes. Digestive vacuoles and apparently coated pits and coated vesicles are also present (Figs. 9, 14). No cytoplasmic crystals were observed.

Granulocytes

About 97% of the hemocytes are granulocytes. They have a heterochromatic nucleus, a poorly developed but distended RER, few free ribosomes, and few mitochondria (Figs. 1, 5). However, their most prominent feature is the many large secretory granules with diameters around 1–2 μm (see below).

Large secretory granules

The majority of the large secretory granules in granulocytes have a uniform content (Fig. 1). However, one class of granules, with structures ranging from amorphous to highly organized tubules with diameters around 17 nm, are seen in close proximity to Golgi complexes (Figs. 7–10). When hemocytes are stimulated with endotoxin a second class of structured granules containing tubules with diameters around 10 nm become transitorily present (Fig. 11). A reverse relationship seems to exist between the numbers of structured granules of the second class and the uniform granules. After this, exocytosis occurs (Fig. 12). In contrast, structured granules of the first class are usually not exocytosed following stimulation with endotoxin (Figs. 13, 14). Following exocytosis, the granulocytes gain numerous pseudopodia, and the organelles collect in the center of the cell surrounded by microtubules (Figs. 13, 14).

Plasmatocytes

Plasmatocytes constitute about 3% of the hemocytes. This conclusion is reached by examining duplicate sam-

Figure 11. Granulocyte from *T. tridentatus* incubated with 10^{-4} g endotoxin per ml hemolymph for 30 s. A stimulated large secretory granule (SG) is in close connection with the plasma membrane (PM). Its tubular structures (arrows) have a diameter around 10 nm, while 17-nm tubular structures (arrowheads) are present in the immature granule (IG) located in close proximity to a Golgi complex (G).

Figure 12. Successive stages in exocytosis of the large secretory granules from *T. tridentatus* granulocytes. Bar length: 500 nm.

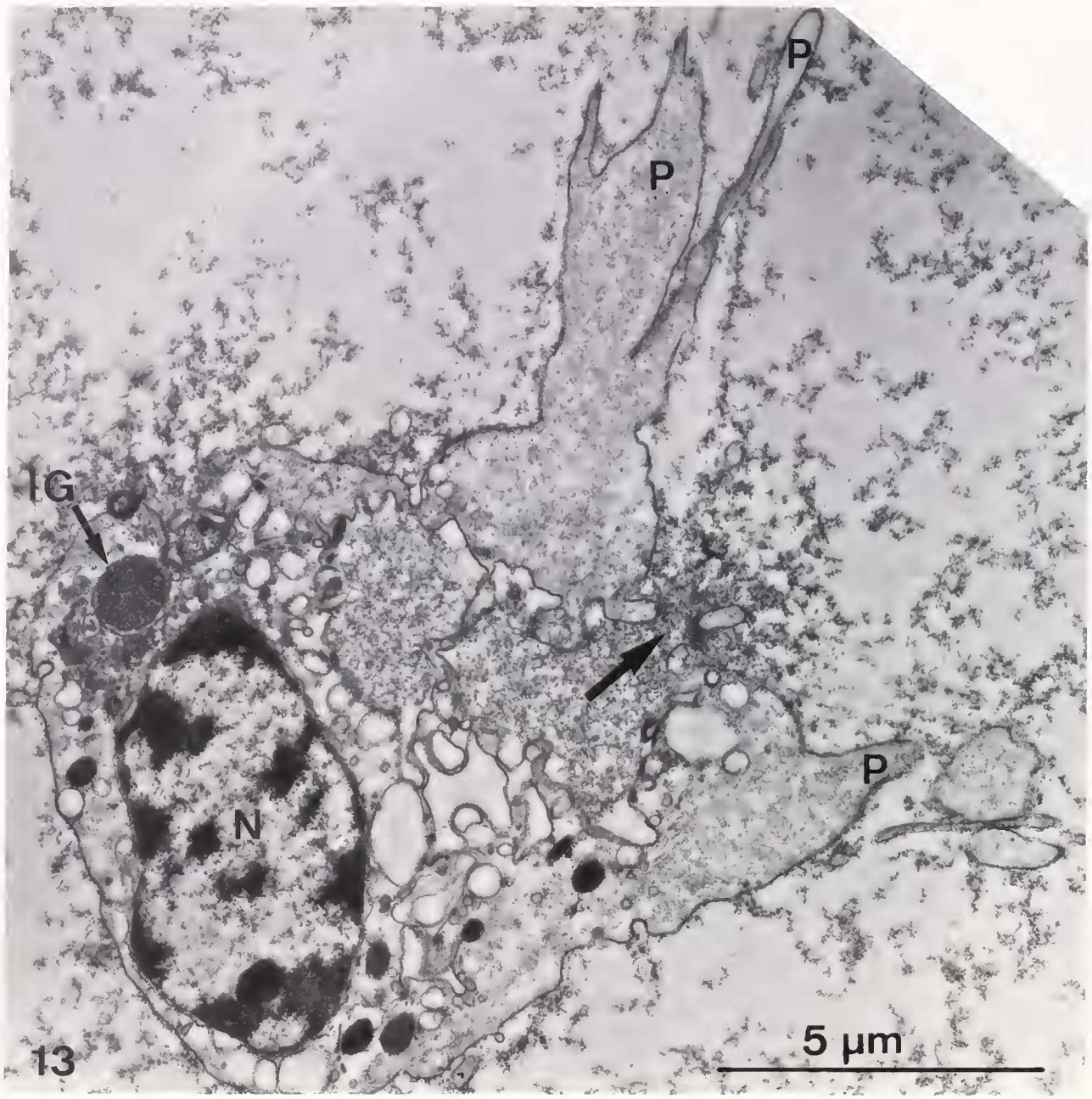


Figure 13. Granulocyte from *Tachypleus tridentatus* incubated with 10^{-4} g endotoxin per ml hemolymph for 300 s. The large secretory granules are exocytosed (arrow), except the immature ones (IG), and pseudopodia (P) are projected. Nucleus (N).

ples from each of six animals. From all samples, at least 10 sections, each containing more than 100 hemocytes, were examined by light microscopy; at least 10 sections were examined by transmission electron microscopy. The plasmatocyte has an euchromatic nucleus, a well-developed system of flattened or tubular cisternae of RER, and many free ribosomes (Figs. 2, 6). Mitochondria are approximately three times as frequent as in granulocytes (Table 1). Plasmatocytes contain few, if any, large secretory granules. These observations are confirmed by LM serial sections of 12 different plasmatocytes (Fig. 15): two plasmatocytes contained zero, five contained one, three contained two, and two contained three large granules. Plasmatocytes are not affected by endotoxin stimulation.

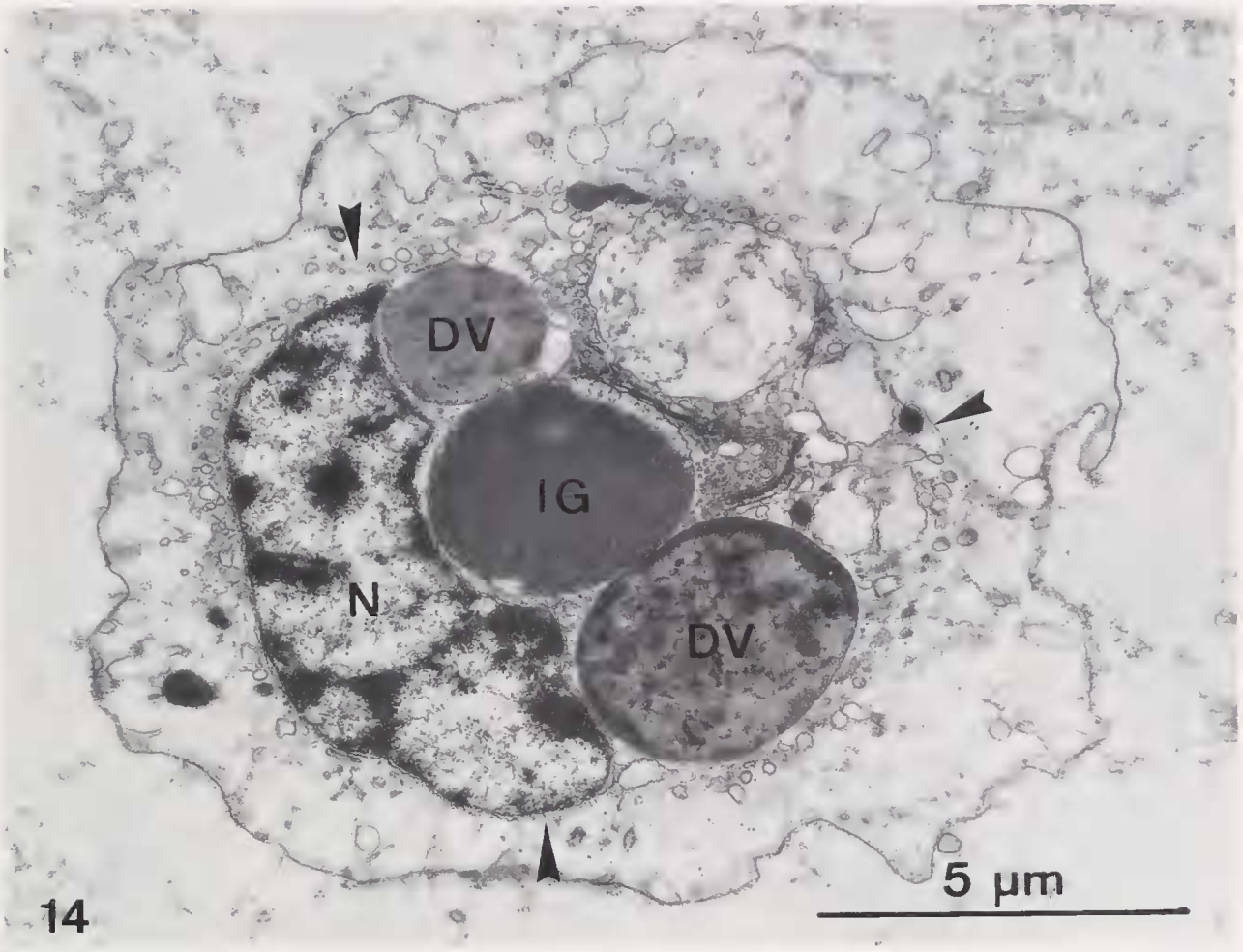


Figure 14. Granulocyte from *Tachypleus tridentatus* incubated with 10^{-4} g endotoxin per ml hemolymph for 300 s. After exocytosis, the remaining organelles collect in the middle of the cell surrounded by microtubules (arrowheads) as observed also in *Limulus polyphemus* (Tablin and Levin, 1988). Digestive vacuoles (DV). Immature granule (IG); nucleus (N).

Discussion

Two major groups of hemocytes

We reveal one granular and one almost agranular type of hemocyte in the general circulation of *T. tridentatus* (Figs. 1, 2, 15). In agreement with the terminology from other arthropods, including other chelicerates, these hemocytes are named granulocytes and plasmatocytes, respectively (Gupta, 1979; Sherman, 1981; Gupta, 1985; Suhr-Jessen *et al.*, 1989). Their main differences are summarized in Table II. The plasmatocyte has not previously been observed in *T. tridentatus*, but it makes up about 3% of the hemocytes in all samples from the six animals studied.

The plasmatocyte is not a cyanoblast or a cyanocyte (Fahrenbach, 1970), because plasmatocytes have the same size, are present in the general circulation of all animals studied at all times, and do not contain cytoplasmic crystals.

The plasmatocyte is not a granulocyte that exocytosed during sampling, because the two cells differ in amounts of hetrochromatin, RER, free ribosomes, and mitochondria (Table II). In other systems, such dramatic changes usually takes hours. Furthermore, the plasmatocyte has the smooth ellipsoidal shape with a marginal band characteristic of the unstimulated granulocyte in contrast to the pseudopodial form following exocytosis (Figs. 1, 13; Dumont *et al.*, 1966; Armstrong, 1980; Armstrong and Rickles, 1982; Armstrong, 1985; Tablin and Levin, 1988). However, it cannot be excluded that plasmatocytes are granulocytes, which have undergone spontaneous exocytosis so early prior to hemolymph sampling that the marginal band of microtubules have reformed. Because the production of gran-

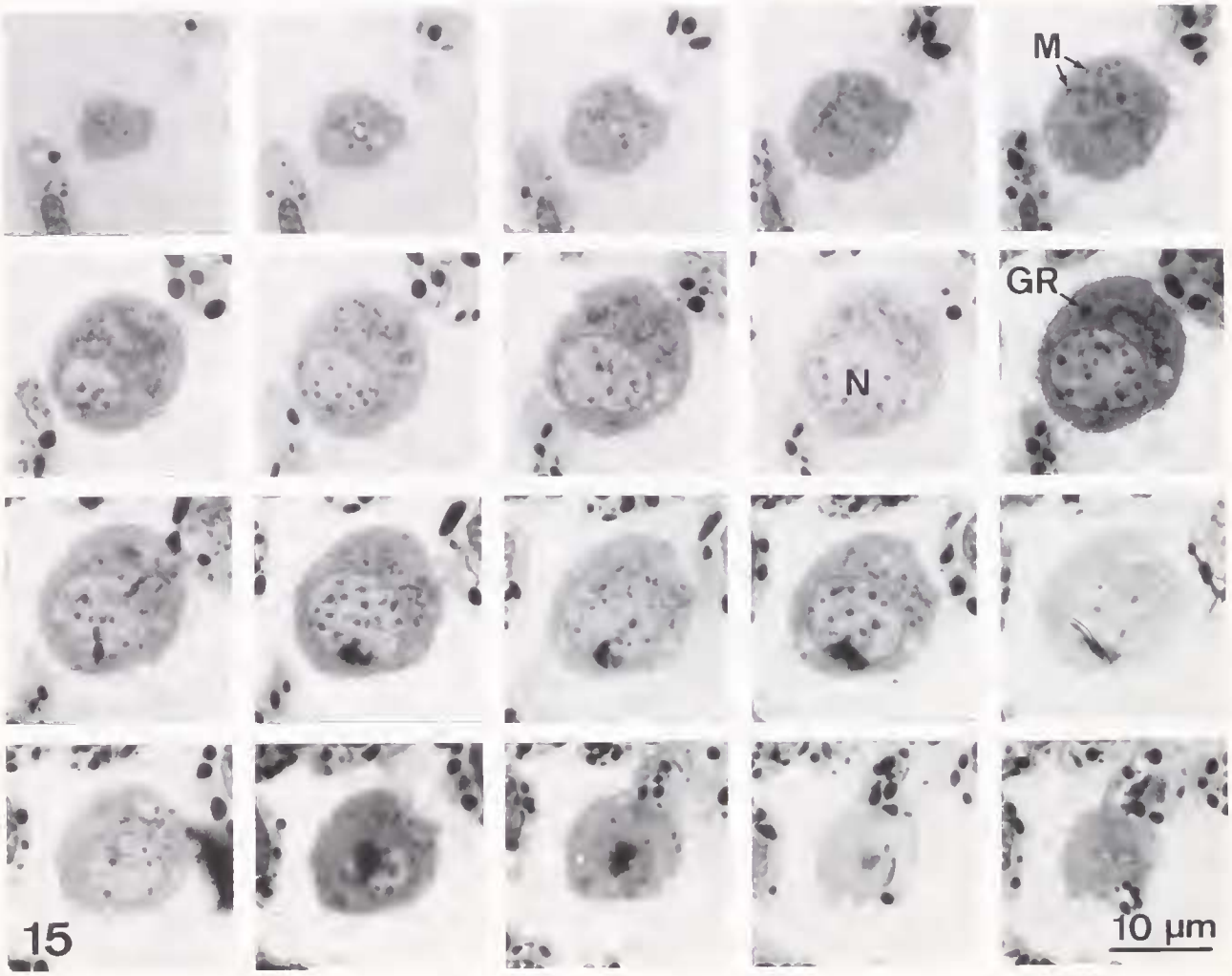


Figure 15. Serial sections of a plasmatocyte from *Tachypleus tridentatus*. The nucleus (N) is uniformly euchromatic; a single large granule (GR) and many mitochondria (M) are present. The neighboring granulocytes contain many large secretory granules, but few mitochondria.

ulocytes is not continuous (Cohen, 1985), this latter interpretation implies either: (1) that approximately 3% of the hemocytes in all *T. tridentatus* examined are constantly recovering from spontaneous exocytosis, and that the transition from plasmatocyte to granulocyte is so fast that intermediate stages are at least one order of magnitude less frequent than plasmatocytes; or (2) that approximately 3% of the hemocytes in each animal recover from a single burst of exocytosis long before the first sampling, and that this recycling is blocked at the plasmatocyte stage. In *L. polyphemus*, the independence of granulocytes and plasmatocytes is further supported by the detection of coagulogen only in granulocytes (Suhr-Jessen *et al.*, 1989).

Large secretory granules

The large structured granules seen in the proximity of Golgi complexes in granulocytes are apparently not affected by endotoxin (Figs. 7–10, 13, 14). This supports the interpretation that this class of structured granules is an immature stage leading to the mature uniform secre-

Table I

A comparison of the abundance of mitochondria in plasmatocytes (PL) and granulocytes (GR) of Tachypleus tridentatus

Serial section	Number of mitochondria in		Ratio PL/GR
	Plasmatocyte	Granulocyte	
#1	154	48	3.25
#2	276	86	3.21
#3	138	51	2.71
Total	568	185	≈3

Each serial section number refers to one plasmatocyte and one granulocyte. Eighteen to 23 serial sections were required to completely section a cell.

Table II

A comparison of the main differences between plasmatocytes and granulocytes in Tachypleus tridentatus

	Plasmatocyte	Granulocyte
Nucleus	Euchromatic	Heterochromatic
RER	Flattened and well developed	Distended but poorly developed
Free ribosomes	Many	Few
Large secretory granules	Few—if any	Many
Mitochondria	Many	Few
Frequency	3%	97%

tory granules, as suggested for *L. polyphemus* (Copeland and Levin, 1985; Suhr-Jessen *et al.*, 1989). Following endotoxin stimulation, the content of the mature uniform granules become transitorily structured before exocytosis (Fig. 11). This resembles the situation in rat mast cells and human platelets (Bloom, 1974; Morgenstern *et al.*, 1987). The different responses to endotoxin suggest that immature and mature secretory granules contain different membrane proteins.

Immune defense

Granulocytes and plasmatocytes from the Asian *T. tridentatus* are cytologically indistinguishable from those in the American *L. polyphemus* (Dumont *et al.*, 1966; Shishikura *et al.*, 1977; Gupta, 1979; Nemhauser *et al.*, 1980; Ornberg and Reese, 1981; Shishikura *et al.*, 1982; Armstrong, 1985; Copeland and Levin, 1985; Tablin and Levin, 1988; Suhr-Jessen *et al.*, 1989). This suggests that the cellular part of their immune defense systems has remained unchanged for more than 140 million years (Shishikura *et al.*, 1982).

Do granulocytes also participate in the endocytic part of the immune defense system, as debated by Armstrong and Levin (1979)? Although digestive vacuoles are present (Fig. 14), we have not observed the formation of large endocytic vacuoles, neither in plasmatocytes nor in granulocytes, but both cells form micropinocytotic (coated) vesicles (Fig. 9). It is tempting to speculate that granulocytes and plasmatocytes may operate together, and with the humoral part of the immune defense system, to recognize and destroy invading microorganisms.

Although the cellular part of the immune defense system in horseshoe crabs has been studied extensively, neither the hemocyte stem cell nor its location, regulation of maturation, differentiation, or proliferation is elucidated (Cohen, 1985). The fate of the granulocytes after exocytosis is also unknown. The gathering of the organelles in the middle of the granulocyte after exocytosis might be the first step in a recovery process (Fig. 14).

The present study describes a hitherto overlooked hemocyte, the plasmatocyte, in the general circulation of *T. tridentatus*. It also extends previous studies of the temporal relationship between maturation and structure of the large secretory granules in granulocytes. Both results prompt several questions pertinent to the molecular biology, structure, and function of hemocytes in horseshoe crabs in particular, and to the evolution and cell biology of the immune defense system in animals in general.

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